Comparisons of the amylolytic enzymes and malt starch hydrolysates of two barley cultivars, Hokudai 1 (the first cultivar developed in Japan) and Kitanohoshi (currently used cultivar for beer production)

Wataru Saburi* and Haruhide Mori

Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan

***Corresponding author:**

Wataru Saburi

Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan

E-mail: saburiw@agr.hokudai.ac.jp

Running head: Amylases and malt starch degradation in Hokudai 1

Abstract

Starch degradation in malted barley produces yeast-fermentable sugars. In this study, we compared the amylolytic enzymes and composition of the malt starch hydrolysates of two barley cultivars, Hokudai 1 (the first cultivar established in Japan) and Kitanohoshi (the currently used cultivar for beer production). Hokudai 1 malt contained lower activity of amylolytic enzymes than Kitanohoshi malt, although these cultivars contained α-amylase AMY2 and β-amylase Bmy1 as the predominant enzymes. Malt starch hydrolysates of Hokudai 1 contained more limit dextrin and less yeast-fermentable sugars than that of Kitanohoshi. In mixed malt saccharification, a high Hokudai 1 malt ratio increased the limit dextrin levels and decreased the maltotriose and maltose levels. Even though Kitanohoshi malt contained more amylolytic enzymes than Hokudai 1 malt, addition of Kitanohoshi extract containing the amylolytic enzymes did not enhance malt starch degradation of Hokudai 1. Hokudai 1 malt starch was less degradable than Kitanohoshi malt starch.

Keywords: barley, starch, amylase, limit dextrin, beer

Introduction

Starch is the major reserved carbohydrate in cereal seeds and is composed of two types of polysaccharides: amylose and amylopectin. Both polysaccharides are polymers of D-glucose mainly linked by α-(1→4)-linkage. Amylose is a linear polysaccharide, whereas amylopectin contains branches of α-(1→6)-linkage. The amylose content of starch from barley (*Hordeum vulgare*) is 22–26%, and the degrees of polymerization (DP) of amylose and amylopectin are approximately 1600 and 5700–7900, respectively (Tang *et al.* 2001).

Degradation of starch in barley seeds is achieved by four types of amylolytic enzymes: α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), α-glucosidase (EC 3.2.1.20), and limit dextrinase (EC 3.2.1.41) (Gupta *et al.* 2010). α-Amylase hydrolyzes the internal α-(1→4)-linkages of starch (MacGregor 1987). β-Amylase and α-glucosidase are exo-type glycoside hydrolases that split the α-(1→4)-linkages of solubilized starch and maltooligosaccharides to produce maltose and D-glucose from non-reducing ends, respectively. Limit dextrinase, also known as pullulanase, hydrolyzes the α-(1→6)-linkages of limit dextrin branches.

Barley contains two β-amylase isozymes: Bmy1 and Bmy2. The genes encoding these isozymes are expressed differently; *Bmy1* and *Bmy2* are expressed in the late and early stages of grain development, respectively (Vinje *et al.* 2011, 2019). The expression level of *Bmy1* is much higher than that of *Bmy2*, and Bmy1 is highly abundant in mature seeds. Therefore, Bmy1 is considered as an endosperm-specific β-amylase (Vinje *et al.* 2011). Other amylolytic enzymes, α-amylase, α-glucosidase, and limit dextrinase, are produced in the aleurone layer cells of the mature seed endosperm via *de novo* protein synthesis in response to gibberellic acid during germination (Hardie 1975). Two α-

amylase isozymes, AMY1 and AMY2, with low and high pI, respectively, are present in barley malt (Marchylo and MacGregor 1983). AMY1 is the major component of embryo-produced α-amylase, whereas AMY2 is produced in the aleurone layers via gibberellic acid stimulation and is the predominant isozyme in barley malt (Macgregor 1987). AMY2 has several isoforms, of which AMY2-1 and AMY2-2 are separated via chromatofocusing (Marchylo and MacGregor 1983; Ajandouz *et al.* 1992). Other AMY2 isoforms with different pI and molecular mass have been identified via proteomic analysis of barley malt protein (Østergaard *et al.* 2002). These isoforms are produced via post-translational modifications during germination. The barley genome contains a single gene encoding the limit dextrinase (Burton *et al.* 1999; Kristensen *et al.* 1999). Gibberellic acid response elements are present in the promoter region of the limit dextrinase gene, and limit dextrinase mRNA is abundant in gibberellic acid-treated aleurone layers (Burton *et al.* 1999). The presence of limit dextrinase in germinating malt has been confirmed via N-terminal amino acid sequence analysis and mass spectrometric analyses of the tryptic peptides of the enzyme purified from germinating malt (Kristensen *et al.* 1999). Moreover, a high pI α-glucosidase encoded by *HvAgl97* is observed in barley malt (Frandsen *et al.* 2000). Transcription of *HvAgl97* is observed in steeping seeds, and the transcription level reaches a maximum of 48 h after steeping. Compared with α-amylase and β-amylase, limit dextrinase and α-glucosidase are less in barley malt.

Degradation of starch granules which accumulate in the endosperm of malted barley seeds is a key process in the production of low-molecular-weight sugars, including maltotriose, maltose, and D-glucose, for alcohol production via yeast fermentation. Starch granules, which are partially solubilized by α-amylase during germination, are degraded by endogenous amylolytic enzymes. Breeding barley cultivars for beer production has

long history, but how the starch degradation properties of malted barley is improved is unclear. Therefore, in this study, we compared the amylolytic enzymes and sugar compositions of the starch hydrolysates of two barley cultivars, Hokudai 1 (the first cultivar established in Japan by Takajiro Minami) and Kitanohoshi (currently used for beer production).

Materials and methods

Dry malt

Dry malts from Hokudai 1 and Kitanohoshi were provided by Sapporo Breweries (Tokyo, Japan). Dry malt was prepared via micromalting, as described by Hoki *et al.* (2018). Barley samples from Hokudai 1 and Kitanohoshi were harvested in Hokkaido and Gunma (Japan), respectively. Phoenix Micromalting Apparatus (Phoenix Biosystems, Edwardstown, SA, Australia) was used with a program for micromalting described below. (1) Steeping: repeated cycles of 6-h immersion and 6-h air-rest at 15 °C. The cast moisture of Hokudai 1 was 43.7 ± 0.4%, and that of Kitanohoshi was 43.8 ± 0.5%. The total steeping time was estimated in advance for each sample using 10 g of each subsample. (2) Germination: total germination time was 144 h, and temperature was 15 °C. (3) Kilning: kilning process involved 100% fresh air, 0% recirculation for 13.5 h at 55 °C, 100 and 0% for 8 h at 65 °C, 50 and 50% for 3.5 h at 75 °C, and 50 and 50% for 4 h at 83.5 °C.

Enzyme extraction

Malt flour was prepared by crushing dry malt in a mill. Malt flour (3 g) was suspended in 20 mL of 50 mM sodium malate buffer (pH 5.4) containing 50 mM NaCl and 0.8 mM

106 $CaCl_2$ and incubated with continuous stirring using a magnetic stirrer at 4 °C for 12 h.
107 The supernatant was obtained using centrifugation at 5,800 × *g* and 4 °C for 10 min.
108
109 **Enzyme assay**
110 β-Amylase activity was determined from the hydrolytic velocity of maltotetraose, a
111 very poor substrate of barley α-amylase (MacGregor 1987). A reaction mixture (50 μL),
112 containing the enzyme, 4 mM maltotetraose (Nihon Shokuhin Kako, Tokyo, Japan), 20
113 mM sodium malate buffer (pH 5.4), 20 mM NaCl, 0.32 mM $CaCl_2$, and 0.2 mg/mL bovine
114 serum albumin (Nacalai Tesque, Kyoto, Japan), was incubated at 37 °C for 10 min. The
115 reaction was stopped by heating the samples at 80 °C for 3 min. Maltose produced was
116 measured as follows: quantification reagent (120 μL), containing 0.44 U/mL maltose
117 phosphorylase (Hidaka *et al.* 2005), 83 mM sodium phosphate buffer (pH 7.0), and
118 glucose CII-test (20 μL; Fujifilm Wako Pure Chemical, Osaka, Japan), was added to the
119 reaction mixture and incubated at 37 °C for 1 h. The maltose concentration was
120 determined by measuring the absorbance at 505 nm (A_{505}).
121 α-Glucosidase activity was determined from the hydrolytic velocity of maltose. The
122 reaction mixture (100 μL), containing the enzyme, 8 mM maltose (Nihon Shokuhin Kako),
123 20 mM sodium malate buffer (pH 5.4), 20 mM NaCl, 0.32 mM $CaCl_2$, and 0.2 mg/mL
124 bovine serum albumin, was incubated at 37 °C for 10 min. The enzyme reaction was
125 stopped by adding 200 μL of 2 M Tris-HCl buffer (pH 7.0), and the D-glucose liberated
126 was measured using the glucose CII-test.
127 α-Amylase and limit dextrinase/pullulanase activities were measured using the α-
128 amylase assay kit (Ceralpha method; Magazyme, Bray, Ireland) and the pullulanase/limit-
129 dextrinase assay kit (PullG6 method; Megazyme), respectively.

One enzyme unit (1 U) was defined as the enzyme amount producing 1 μmol of reaction product in 1 min.

Measurement of protein

Concentration of purified α-amylase and β-amylase was determined from A_{280} using the specific coefficient for 1 mg/mL solution, estimated according to Pace *et al.* (1995): specific coefficient of α-amylase (AMY2, NCBI reference sequence: XP_044955531.1), 2.46; and that of β-amylase (Bmy1, GenBank accession number: QBQ34099.1), 1.55.

Starch degradation in malt

Malt flour (200 mg), prepared as described above, was suspended in 0.6 mL of 50 mM sodium acetate buffer (pH 5.5) and incubated at 65 °C for 90 min. The sample was incubated at 100 °C for 10 min to stop the reaction. The supernatant was obtained via centrifugation at 20,000 × *g* and 4 °C for 5 min and diluted 5-fold with water. Then, the sample was desalted with an ion exchange resin, Amberlite MB-4 (Organo, Tokyo, Japan), and passed through a 0.45-μm membrane filter (Advantec, Tokyo, Japan). Then, 10 μL of the sample was analyzed using high performance liquid chromatography (HPLC) under the following conditions: Aminex HPX-42A column (7.8 mm I.D. × 300 mm; Bio-Rad, Hercules, CA, USA); column temperature, 75 °C; elution, water; flow rate, 0.5 mL/min; detection, refractive index. For the reaction of mixed malt, the malt flours of Hokudai 1 and Kitanohoshi were mixed before adding the buffer. Malt saccharification in the presence of bacterial pullulanase was performed by adding 0.5 μL of 491 U/mL *Klebsiella* pullulanase (Pullulanase Amano 3; Amano Enzyme, Nagoya, Japan; 0.4 U/mL in the reaction mixture).

154 The degradation of Hokudai 1 starch by the amylolytic enzymes from Kitanohoshi
155 malt was carried out as follows: 200 mg of malt flour from Hokudai 1 and Kitanohoshi
156 was suspended in 1 mL of 50 mM sodium acetate buffer (pH 5.5), and the precipitate and
157 supernatant (extract) were separated immediately via centrifugation at 20,000 × *g* and
158 4 °C for 5 min. The precipitated fraction of Hokudai 1 malt was suspended in the extract
159 of Kitanohoshi malt flour and reacted as described above.
160
161 **Structural analyses of limit dextrins derived from the malt starch of Hokudai 1 and**
162 **Kitanohoshi**
163 Malt starch hydrolysate was prepared as described above, but 10 g of malt flour
164 was used here. To reduce the fermentable carbohydrates, dry yeast (250 mg; Nisshin Flour
165 Milling, Tokyo, Japan) was added to the sample and incubated with vigorous shaking at
166 30 °C for 40 h. The supernatant, obtained via centrifugation (2,300 × *g* at 4 °C for 10
167 min), was separated via gel filtration column chromatography under the following
168 conditions: Toyopearl HW-40S column (5 cm I.D. × 110 cm; Tosoh, Tokyo, Japan);
169 elution, water; flow rate, 0.5 mL/min; fraction volume, 10 mL. The concentrations of total
170 and reducing sugars were measured using the phenol sulfuric acid (Dubois *et al.* 1956)
171 and copper bicinchoninate (Fox and Robyt 1991) methods, respectively, with D-glucose
172 as the standard carbohydrate. DP was estimated from the concentrations of total and
173 reducing sugars. Fractions containing sugars with average DPs of 26, 15, and 10 were
174 collected, desalted, and lyophilized. The prepared polysaccharide sample was treated with
175 pullulanase to confirm the presence of limit dextrin containing the α-(1→6)-branch. A
176 mixture containing 5 mg/mL polysaccharide, 20 mM sodium acetate buffer (pH 5.5), and
177 0.8 U/mL of the pullulanase was incubated at 37 °C for 90 min. The reaction was stopped

by heating the sample at 100 °C for 5 min, and sugar composition was analyzed via HPLC, as described above. Nuclear magnetic resonance (NMR) spectra were recorded in D_2O at 60 °C using a Bruker Avance Neo (500 MHz; Bruker Corporation, Billerica, MA, USA).

Identification of α-amylase and β-amylase from Hokudai 1 and Kitanohoshi

Enzyme extraction from 20 g of malt flour was performed as described above, and the extract was dialyzed against 10 mM HEPES-NaOH buffer (pH 7.0). The resulting samples were separated on the DEAE Sepharose Fast Flow column (2.6 cm I.D. × 10 cm; Cytiva, Uppsala, Sweden) equilibrated with 10 mM HEPES-NaOH buffer (pH 7.0). After the elution of non-adsorbed proteins, the adsorbed proteins were eluted using a 0–1 M NaCl linear gradient (400 mL in total). The volumes of non-adsorbed and adsorbed fractions were 15 and 5 mL, respectively. Fractions containing α-amylase and β-amylase were collected, and α-amylase was purified via β-cyclodextrin (β-CD) Sepharose 6B column chromatography (1.6 cm I.D. × 7 cm). β-CD Sepharose 6B was prepared as previously described (Vretblad 1974). The column was equilibrated with 10 mM sodium acetate buffer (pH 6.0) containing 5 mM $CaCl_2$ and 50 mg/mL ammonium sulfate. After washing the column with the equilibrium buffer, the adsorbed protein was eluted with the equilibrium buffer containing 10 mg/mL β-CD. β-Amylase was purified from the non-adsorbed fractions of the β-CD Sepharose 6B column chromatography via hydrophobic column chromatography using the Toyopearl butyl-650M column (1.6 cm I.D. × 6 cm; Tosoh) and gel filtration column chromatography using the Toyopearl HW-50S column (1.6 cm I.D. × 60 cm; Tosoh). The Toyopearl butyl-650M column was equilibrated with 10 mM HEPES-NaOH buffer (pH 7.0) containing 30% saturation ammonium sulfate, and elution of the adsorbed protein was performed using a descending linear gradient of

ammonium sulfate (30–0% saturation; 200 mL in total). The Toyopearl HW-50S column was equilibrated with 10 mM HEPES-NaOH buffer (pH 7.0) containing 0.1 M NaCl.

The purified protein (2 μg) was separated via SDS-PAGE. The band of target protein was excised, and the protein was digested with trypsin using the In-Gel Tryptic Digestion Kit (Thermo Fisher Scientific, Waltham, MA, USA). The resulting peptides were purified using the Pierce C18 Spin Column (Thermo Fisher Scientific) and diluted 20 times with 0.1% (v/v) formic acid. The sample (2 μL) was analyzed using LC-MS/MS EASY-nLC1000 (Thermo Fisher Scientific) under the following conditions: NTCC-360/75-3-125 C18 column (0.075 mm I.D. × 120 mm; Nikkyo Technos, Tokyo, Japan), elution, 0–90% (v/v) acetonitrile in 0.1% (v/v) formic acid (acetonitrile concentration: 0 min, 0%; 1 min, 5%; 21 min, 35%; 23 min, 90%; 40 min, 90%); flow rate, 300 nL/min; mass spectrometer, Orbitrap Elite Hybrid Ion Trap-Orbitrap Mass Spectrometer (Thermo Fisher Scientific Scientific). The data were processed using the search software Proteome Discoverer (Thermo Fisher Scientific).

Results

Comparison of the amylolytic enzyme activities of Hokudai 1 and Kitanohoshi

Activities of the amylolytic enzymes α-amylase, β-amylase, α-glucosidase, and pullulanase in the dry malt extracts of Hokudai 1 and Kitanohoshi were measured (Table 1). Activities of α-amylase (115 U) and β-amylase (180 U) were high in 1 g of Hokudai 1 malt, and they were the predominant amylolytic enzymes similar to those in Kitanohoshi malt; however, the activity in Hokudai 1 malt was approximately 80% of that in Kitanohoshi malt. Activities of α-glucosidase (0.693 U) and limit dextrinase (0.00356 U) in 1 g of Hokudai 1 malt were 87% and 63% of those in Kitanohoshi malt,

226 respectively.

227

228 **Comparison of the malt starch hydrolysate compositions of Hokudai 1 and**
229 **Kitanohoshi**

230 The carbohydrate compositions of the malt starch hydrolysates of Hokudai 1 and
231 Kitanohoshi were analyzed (Figure 1a and b). The sugar composition plateaued during
232 the reaction period (90 min). Carbohydrate profiles in the malt starch hydrolysates of the
233 two barley cultivars were similar, but in the starch hydrolysate of Hokudai 1 malt, the
234 relative content of polysaccharides with >9 DP was higher (Hokudai 1, 16.8%;
235 Kitanohoshi, 12.7%), whereas that of DP3 (maltotriose; Hokudai 1, 11.2%; Kitanohoshi,
236 13.1%) and DP2 (maltose; Hokudai 1, 50.7%; Kitanohoshi, 52.7%) was lower than that
237 of Kitanohoshi malt. Saccharification of the mixed malt of Hokudai 1 and Kitanohoshi
238 showed a good linear relationship between the ratio of Hokudai 1 malt and the contents
239 of fermentable sugars, polysaccharides, maltotriose, and maltose (Figure 1c–h). The high
240 Hokudai 1 malt ratio decreased the contents of fermentable sugars, maltotriose, and
241 maltose and increased the polysaccharide content. The increase in polysaccharide content,
242 along with an increase in the Hokudai 1 malt ratio, was roughly consistent with the
243 decrease in maltotriose and maltose contents.

244 To degrade starch in Hokudai 1 malt using amylolytic enzymes from Kitanohoshi
245 malt, an insoluble material containing starch in a suspension of Hokudai 1 malt flour was
246 incubated with the extract of Kitanohoshi malt containing amylolytic enzymes. The sugar
247 content of the resulting starch hydrolysate was almost identical to that of the hydrolysate
248 prepared using the original enzymes (Figure 1i), although Kitanohoshi malt contained
249 more amylolytic enzymes than Hokudai 1 malt, as described above.

Malt starch degradation was observed in the presence of bacterial pullulanase (Figure 1j). The addition of pullulanase decreased the relative polysaccharide content (Hokudai 1, 17.0→7.23%; Kitanohoshi, 12.8→6.31%) and increased the relative contents of maltotriose (Hokudai 1, 12.2→16.1%; Kitanohoshi, 13.8→16.6%) and maltose (Hokudai 1, 48.9→57.8%; Kitanohoshi, 50.9→57.1%) in both Hokudai 1 and Kitanohoshi malts. The sugar compositions of the saccharified malt starch of the two barley cultivars were similar in the presence of pullulanase.

Structural analyses of the limit dextrins of Hokudai 1 and Kitanohoshi

Polysaccharides remaining after the saccharification of Hokudai 1 and Kitanohoshi malt starches were subjected to gel filtration column chromatography (Figure 2a). Three and two polysaccharide samples with different DPs were obtained from the starch hydrolysates of Hokudai 1 and Kitanohoshi, respectively. The average DPs of polysaccharides 1, 2, and 3 from Hokudai 1 were 26, 15, and 10, respectively, whereas those of polysaccharides 1 and 2 from Kitanohoshi were 26 and 15, respectively. Polysaccharides 2 and 3 of Hokudai 1 and polysaccharide 2 of Kitanohoshi were almost completely degraded into oligosaccharides by pullulanase, and the HPLC elution profiles of these samples were similar (Figure 2b). Polysaccharide 1 was partially degraded in both barley cultivars. These results indicate that the polysaccharide samples of these barley cultivars with high DPs contained polysaccharides other than limit dextrin, and the polysaccharide with 10–15 DP was mainly limit dextrin, which predominantly contained branches with DP2 and DP3, as observed in the HPLC chromatogram. The structures of polysaccharides 2 and 3 from Hokudai 1 and polysaccharide 2 from Kitanohoshi were analyzed using quantitative ^{1}H-NMR (Figure 2c). Relative signal intensity for H-1 of the

D-glucosyl residue linking to the 6-position of the reducing end side D-glucosyl residue (0.22; D-glucosyl residue linking to the 4-position of the reducing end side D-glucosyl residue, 1.00) was similar among the three polysaccharide samples, indicating that these polysaccharide samples had similar frequencies of the α-(1→6)-branch. The DPs of polysaccharides 2 and 3 of Hokudai 1 and polysaccharide 2 of Kitanohoshi were estimated to be 12.2, 10.2, and 12.2, respectively, from the relative signal intensity ratio of the reducing end D-glucose residue (polysaccharide 2 of Hokudai 1, 0.10; polysaccharide 3 of Hokudai 1, 0.12; polysaccharide 2 of Kitanohoshi, 0.10) and other D-glucose residues (1.22). These DPs are similar to those described above.

Identification of α-amylase and β-amylase from Hokudai 1 and Kitanohoshi

The elution profiles of the main amylolytic enzymes, α-amylase and β-amylase, in Hokudai 1 and Kitanohoshi malts were compared using anion exchange column chromatography. As shown in panels a and b of Figure 3, the two enzymes from the two barley cultivars were similarly eluted via the column chromatography. The active peak of α-amylase was eluted at approximately 0.1 M NaCl, and the main active peak of β-amylase was eluted at this NaCl concentration. From the active fractions containing both α-amylase and β-amylase, these enzymes were purified to a single band via SDS-PAGE (Figure 3c). The specific activity of α-amylase from Hokudai 1 and Kitanohoshi was 85.6 and 124 U/mg, respectively. The specific activity of β-amylase from Hokudai 1 and Kitanohoshi was 132 and 93.9 U/mg, respectively. Protein sequences of the purified enzymes were analyzed using LC-MS/MS analysis of tryptic peptides. α-Amylase from Hokudai 1 and Kitanohoshi matched the barley α-amylase isozyme, AMY2 (XP_044955531.1), with coverages of 73.9 and 72.2% of 403 amino acid residues of the

298 mature sequence, respectively (Figure 3d). β-Amylase from Hokudai 1 and Kitanohoshi
299 matched the barley β-amylase, Bmy1 (QBQ34099.1), with coverages of 72.0 and 75.7%
300 of 535 amino acid residues of the entire sequence, respectively (Figure 3e).
301
302 **Discussion**
303 The degradation of starch granules in malted barley is a key step in the production
304 of low-molecular-weight sugars for yeast fermentation to produce alcohols. In this study,
305 amylolytic enzymes in malt and sugar composition after starch degradation were
306 compared between two barley cultivars, Hokudai 1 and Kitanohoshi, to determine how
307 starch degradation has been improved during barley breeding for beer production. Dry
308 malts of both cultivars exhibited higher activities of α-amylase and β-amylase than those
309 of α-glucosidase and limit dextrinase. Hokudai 1 malt exhibited slightly lower activities
310 of all amylolytic enzymes compared to Kitanohoshi malt under the analytical conditions
311 in this study (Table 1). LC-MS/MS analysis of the tryptic peptides of α-amylase and β-
312 amylase purified from the two barley malts indicated that these enzymes were encoded
313 by the homologous genes of AMY2 and Bmy1, which are the dominant amylolytic
314 enzymes in barley malt (MacGregor 1987; Vinje *et al.* 2011), respectively (Figure 3).
315 However, the specific activities of α-amylase and β-amylase in the two barley cultivars
316 were different from each other; the specific activity of α-amylase from Hokudai 1 was
317 only 69% of that from Kitanohoshi, whereas the specific activity of β-amylase from
318 Hokudai 1 was 140% of that from Kitanohoshi. This difference in the enzyme activities
319 of the two barley cultivars may be due to differences in post-translational modifications.
320 As a few peptide fragments were detected only in the enzymes derived from one barley
321 cultivar (Figure 3), amino acid residues in the undetected peptides were possibly modified

322 via post-translational modifications such as *O*-glycosylation of the Ser residues included
323 in the undetected peptides, thus affecting the specific activities of the enzymes.

324 The hydrolysate of Hokudai 1 malt starch contained more polysaccharides, including
325 limit dextrin, with >9 DP and less fermentable carbohydrates than that of Kitanohoshi
326 malt starch. No obvious structural difference was observed in the limit dextrin from
327 Hokudai 1 and Kitanohoshi malt starch (Figure 2). In the saccharification of mixed malts
328 of Hokudai 1 and Kitanohoshi, a higher Hokudai 1 malt ratio increased the remaining
329 polysaccharide levels and decreased the maltotriose and maltose levels, but degradation
330 of starch in Hokudai 1 malt was not enhanced by using amylolytic enzymes from
331 Kitanohoshi malt, which contained more limit dextrinase activity than Hokudai 1 malt
332 (the amount of limit dextranase was too low to degrade limit dextrin during the
333 saccharification reaction). These results suggest that the difference in the sugar
334 compositions of the malt starch hydrolysates of the two barley cultivars arises from
335 differences in the structures of malt starches rather than from differences in their
336 amylolytic enzyme activities. Amylopectin of starch in Hokudai 1 malt contains more
337 branches than that in Kitanohoshi, and it might be less degradable by the amylolytic
338 enzymes in barley malt.

339 In this study, we demonstrated that the malt starch of the old barley cultivar, Hokudai
340 1, was less degradable by amylolytic enzymes than that of the currently used barley
341 cultivar, Kitanohoshi. This may be due to the selection of barley cultivars producing
342 starch granules more easily degraded by amylolytic enzymes, along with a long history
343 of breeding barley for beer production. The low degradability of malt starch in Hokudai
344 1 may be due to the low degradation of branches by limit dextrinase during germination
345 for malt production and/or high branch frequency in the starch granules. Therefore, future

346 studies should compare the activity profiles of amylolytic enzymes during germination
347 and the structures of starch granules in Hokudai 1 and currently used barley cultivars,
348 including Kitanohoshi, to better understand the starch degradation process in barley.
349
350 **Acknowledgments**
351 We are grateful to Mr. Daichi Nakatsuka of Sapporo Breweries for providing the
352 Hokudai 1 and Kitanohoshi malts used in this study. We thank Dr. Kan Yamashiro of
353 Amano Enzyme for giving us the bacterial pullulanase. We would like to thank Dr. Eri
354 Fukushi of the GC-MS and NMR Laboratory (Research Faculty of Agriculture, Hokkaido
355 University) for assistance with the NMR analysis.
356
357 **Conflict of Interest**
358 The authors declare no conflicts of interest.
359
360 **Data availability**
361 All data discussed in this article is available upon request from the corresponding author.
362
363 **Authors' contributions**
364 W. S. performed the experiments and wrote the manuscript. H.M. reviewed and edited
365 the manuscript.
366
367 **Funding**
368 This work was supported by Hokkaido University Frontier Foundation [PKF3421005
369 and PKF3422002].

370 **References**

371 Ajandouz EH, Abe J, Svensson B, et al. Barley malt-α-amylase. Purification, action
372 pattern, and subsite mapping of isozyme 1 and two members of the isozyme 2
373 subfamily using p-nitrophenylated maltooligosaccharide substrates. *Biochim Biophys*
374 *Acta* 1992;1159:193–202.

375 Burton RA, Zhang XQ, Hrmova M, et al. A single limit dextrinase gene is expressed both
376 in the developing endosperm and in germinated grains of barley. *Plant Physiol*
377 1999;119:859–871.

378 Dubois M, Gilles KA, Hamilton, JK, et al. Colorimetric method for determination of
379 sugars and related substances. *Anal Chem* 1956;28:350–356.

380 Frandsen TP, Lok F, Mirgorodskaya E, et al. Purification, enzymatic characterization, and
381 nucleotide sequence of a high-isoelectric-point α-glucosidase from barley malt. *Plant*
382 *Physiol* 2000;123:275–286.

383 Fox JD, Robyt JF. Miniaturization of three carbohydrate analyses using a microsample
384 plate reader. *Anal Biochem* 1991;195:93–96.

385 Gupta M, Abu-Ghannam N, Gallaghar E. Barley for brewing: characteristic changes
386 during malting, brewing and applications of its by-products. *Compr Rev Food Sci Food*
387 *Saf* 2010;9:318–328.

388 Hardie GD. Control of carbohydrase formation by gibberellic acid in barley endosperm.
389 *Phytochemistry* 1975;14:1719–1722.

390 Hidaka Y, Hatada Y, Akita M, et al. Maltose phosphorylase from a deep-sea *Paenibacillus*
391 sp.: Enzymatic properties and nucleotide and amino-acid sequences. *Enzyme Microb*
392 *Technol* 2005;37:185–194.

393 Hoki T, Kanatani R, Saito W, et al. Breeding of lipoxygenase-1-less malting barley variety

394 'Satuiku 2 go'. J Inst Brew 2018;124:112–120.
395 Kristensen M, Lok F, Planchot V, et al. Isolation and characterization of the gene encoding
396 the starch debranching enzyme limit dextrinase from germinating barley. Biochim
397 Biophys Acta 1999;1431:538–546.
398 MacGregor AW. α-Amylase, limit dextrinase, and α-glucosidase enzymes in barley and
399 malt. *Crit Rev Biotechnol* 1987;5:117–128.
400 Marchylo BA, MacGregor AW. Separation of barley malt alpha-amylase by
401 chromatofocusing. *Cereal Chem* 1983;60:311–314.
402 Tang H, Ando H, Watanabe K, et al. Physicochemical properties and structure of large,
403 medium and small granule starches in fractions of normal barley endosperm.
404 *Carbohydr Res* 2001;330:241–248.
405 Østergaard O, Melchior S, Roepstorff P, et al. Initial proteome analysis of mature barley
406 seeds and malt. *Proteomics* 2002;2:733–739.
407 Pace CN, Vajdos F, Fee L, et al. How to measure and predict the molar absorption
408 coefficient of a protein. *Protein Sci.* 1995;4:2411–2423.
409 Vinje MA, Willis DK, Duke SH, et al. Differential expression of two β-amylase genes
410 (Bmy1 and Bmy2) in developing and mature barley grain. *Planta* 2011;233:1001–
411 1010.
412 Vinje MA, Walling JG, Henson CA, et al. Comparative gene expression analysis of the
413 β-amylase and hordein gene families in the developing barley grain. *Gene*
414 2019;693:127–136.
415 Vretblad P. Immobilization of ligands for biospecific affinity chromatography via their
416 hydroxyl groups. The cyclohexaamylose-β-amylase system. *FEBS Lett* 1974;47:86–
417 89.

418

419

Figure captions

Figure 1. Sugar composition analysis of malt starch hydrolysate

(a) High-performance liquid chromatography (HPLC) chromatogram of the malt starch hydrolysates of Hokudai 1 and Kitanohoshi. G, D-glucose; F, D-fructose; PS, polysaccharide. Numbers above peaks indicate the degree of polymerization (DP). (b) Relative contents of the carbohydrate components of malt starch hydrolysates of Hokudai 1 and Kitanohoshi. Relative content was calculated from the relative peak area of the HPLC chromatogram shown in panel (a). Black and white bars indicate the Hokudai 1 and Kitanohoshi cultivars, respectively. FC, fermentable carbohydrate (sum of contents of DP3, DP2, D-glucose, and D-fructose). (c–h) Relative contents of the carbohydrate components of the saccharified starch of mixed malt of Hokudai 1 and Kitanohoshi. (c) Fermentable carbohydrates. (d) Polysaccharides. (e) Sum of the carbohydrate contents of DP4–9, (f) maltotriose, (g) maltose, and (h) D-glucose. (i) Saccharification of Hokudai 1 malt with amylolytic enzymes from Kitanohoshi malt. Black and white bars indicate the saccharification with enzymes from Hokudai 1 (control) and Kitanohoshi, respectively. (j) Saccharification of malt starch in the presence of bacterial pullulanase. Black/white and dark/light gray bars indicate the saccharification of malt starch in Hokudai 1 and Kitanohoshi, respectively. Black/dark gray and white/light gray bars indicate the reactions without and with bacterial pullulanase, respectively. Values and error bars represent the average and standard deviation for triplicate independent experiments, respectively.

Figure 2. Structural analysis of the limit dextrins

(a) Elution profile of the saccharified malt starch treated with yeast in gel filtration column chromatography. Fractions indicated in the figure were collected: polysaccharides

1–3 in Hokudai 1 and polysaccharides 1 and 2 in Kitanohoshi. (b) HPLC analysis of the prepared polysaccharide samples before (solid line) and after (dashed line) pullulanase treatment. DP is shown above the peak. (c) Quantitative ^{1}H-NMR analysis of Hokudai 1 polysaccharides 2 and 3, and Kitanohoshi polysaccharide 2. Glcα→4, D-glucosyl residue linking to the 4-position of the reducing end side D-glucosyl residue; Glcα→6, D-glucosyl residue linking to the 6-position of the reducing end side D-glucosyl residue; Glcα, reducing end α-D-glucose residue; Glcβ, reducing end β-D-glucose residue. Values indicate the relative signal intensity.

Figure 3. Identification of α-amylase and β-amylase

(a and b) Elution profiles of the malt extracts of Hokudai 1 and Kitanohoshi, respectively, obtained via DEAE Sepharose Fast Flow column chromatography. Black circles, absorbance at 280 nm; white circles, α-amylase activity; white triangles, β-amylase activity; black triangles, NaCl concentration. Collected fractions are indicated by gray dotted lines. (c) SDS-PAGE analysis of the purified enzymes (1 μg). Proteins were detected via Coomassie Brilliant Blue staining. Lanes K and H indicate the enzymes from Kitanohoshi and Hokudai 1, respectively. (d and e) Amino acid sequences determined using LC-MS/MS analysis of the tryptic peptides of α-amylase (d) and β-amylase (e) purified from Hokudai 1 and Kitanohoshi malts. Blue letters, sequences detected only in the sample of Hokudai 1; red letters, sequences detected only in the sample of Kitanohoshi; purple, sequences detected in both barley cultivars; gray letters, undetected sequences. The secretory signal sequence is underlined.

468 Graphical abstract caption

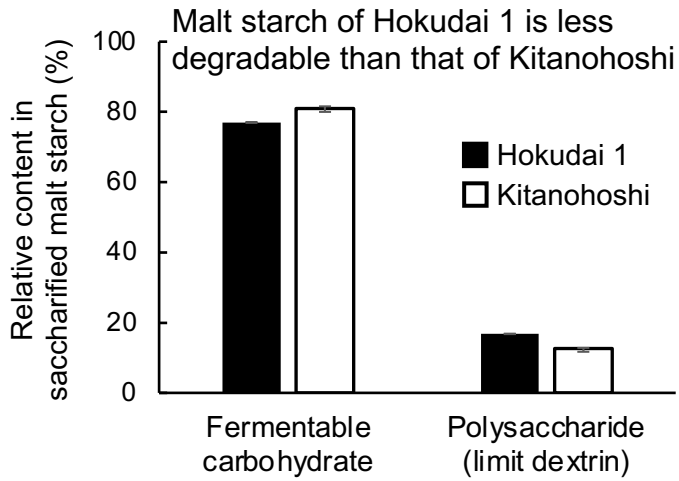
469 Sugar composition of malt starch hydrolysate of barley cultivars, Hokudai 1 (The first

470 cultivar developed in Japan) and Kitanohoshi (currently used cultivar for beer production).

Hokudai 1
The first cultivar developed in Japan

Breeding

Kitanohoshi
Current cultivar for beer production



GA., Saburi et al.

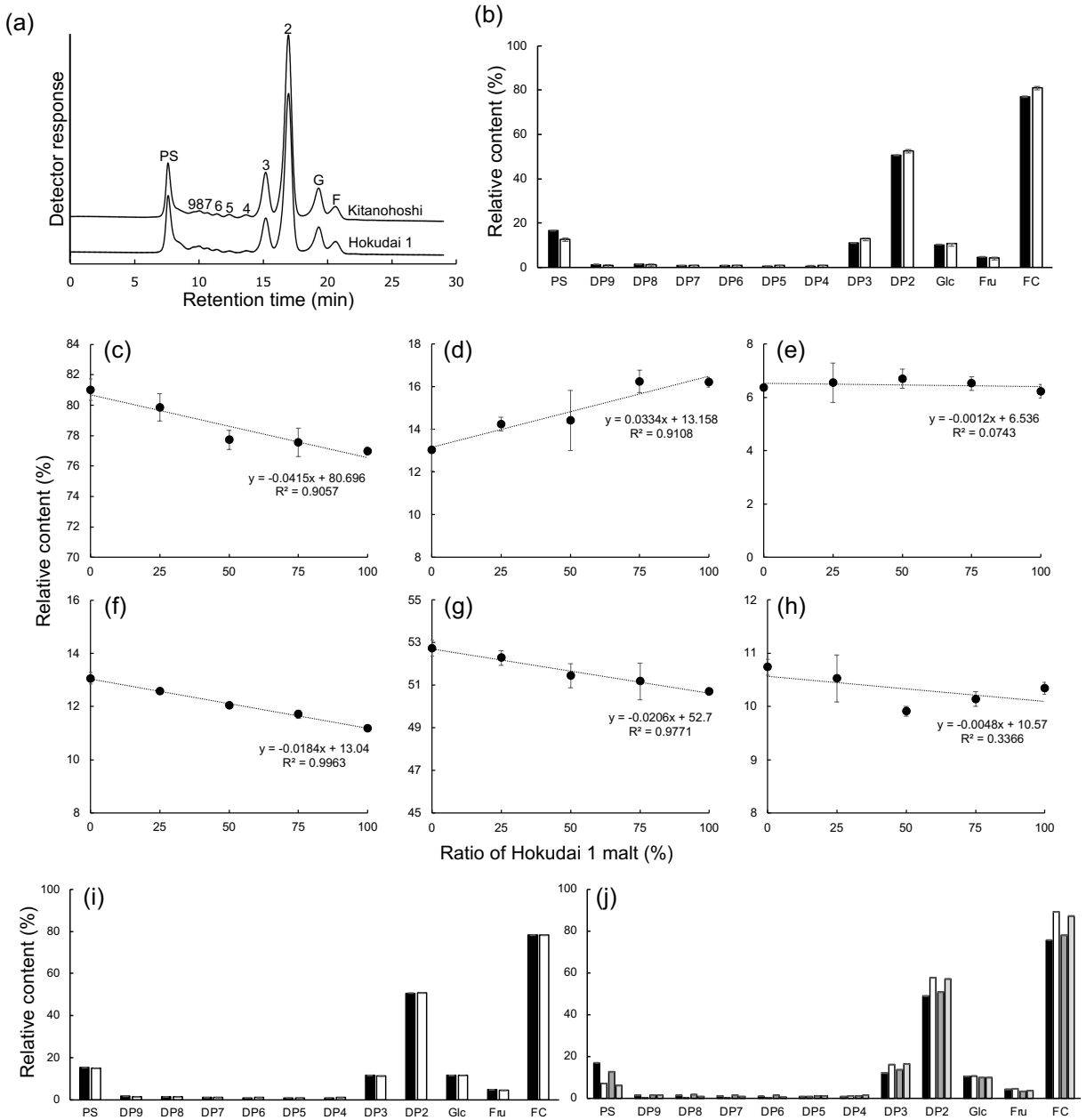


Figure 1., Saburi et al.

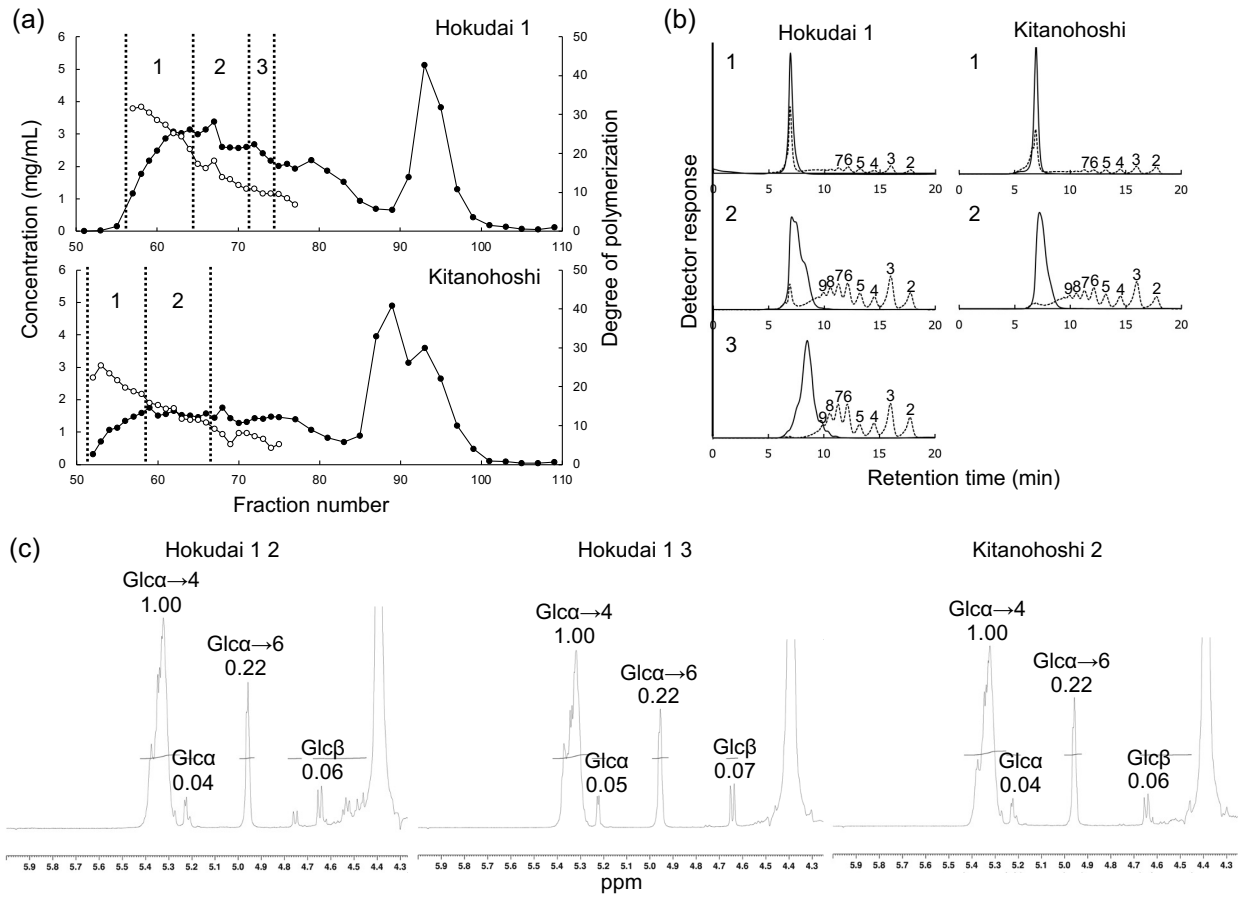
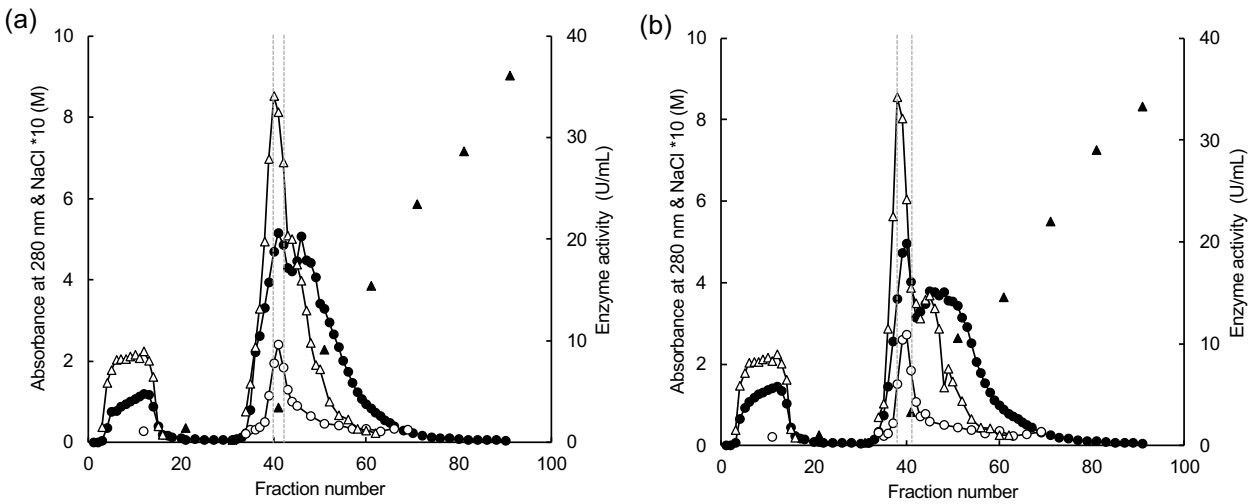
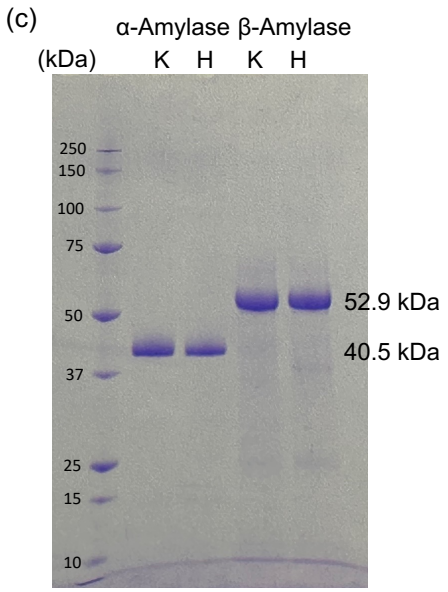


Figure 2., Saburi et al.





(d)

MANKHLSLSLFLVLLGLSASLASGQVLFQGFNWESWKHNGGWYNFLMGKVDDIA
AAGITHVWLPPASQSVAEQGYMPGRLYDLDASKYGNKAQLKSLIGALHGKGVKA
IADIVINHRTAEHKDGRGIYCIFEGGTPDARLDWGPHMICRDDRPYADGTGNPD
TGADFGAAPDIDHLNLRVQKELVEWLNWLKADIGFDGWRFDFAKGYSADVAKIY
IDRSEPSFAVAEIWTSLAYGGDGKPNLNQDQHRQELVNWVDKVGSKGPATTFDF
TTKGILNVAVEGELWRLRGTDGKAPGMIGWWPAKAVTFVDNHDTGSTQHMWPFP
SDRVMQGYAYILTHPGTPCIFYDHFFDWGLKEEIDRLVSVRTRHGIHSESKLQI
IEADADLYLAEIDGKVIVKLGPRYDVGNLIPGGFKVAAHGNDYAVWEKI

(e)

MEVNVKGNYVQVYVMLPLDAVSVNNRFEKGDELRAQLRKLVEAGVDGVMVDVWW
GLVEGKGPKAYDWSAYKQLFELVQKAGLKLQAIMSFHQCGGNVGDAVNIPIPQW
VRDVGTRDPDIFYTDGHGTRNIEYLTLGVDNQPLFHGRSAVQMYADYMTSFREN
MKDFLDAGVIVDIEVGLGPAGEMRYPSYPQSHGWSFPGIGEFICYDKYLQADFK
AAAAAVGHPEWEFPNDAGQYNDTPERTQFFRDNGTYLSEKGRFFLAWYSNNLIK
HGDRILDEANKVFLGYKVQLAIKISGIHWWYKVPSHAAELTAGYYNLHDRDGYR
TIARMLKRHRASINFTCAEMRDSEQSSQAMSAPEELVQQVLSAGWREGLNVACE
NALPRYDPTAYNTILRNARPHGINQSGPPEHKLFGFTYLRLSNQLVEGQNYVNF
KTFVDRMHANLPRDPYVDPMAPLPRSGPEISIEMILQAAQPKLQPFPFQEHTDL
PVGPTGGMGGQAEGPTCGMGGQVKGPTGGMGGQAEDPTSGIGGELPATM

Figure 3., Saburi et al.

Table 1. Activity of amylolytic enzymes in dry malt of Hokudai 1 and Kitanohoshi (U/g malt).

Enzyme	Hokudai 1	Kitanohoshi
α-Amylase	115 ± 3	147 ± 3
β-Amylase	180 ± 2	225 ± 4
α-Glucosidase	0.693 ± 0.058	0.797 ± 0.119
Limit dextrinase	0.00356 ± 0.00024	0.00568 ± 0.00057

Values are average ± standard deviation for triplicated independent experiments.